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Inhibition of Gene Expression by the $^{G}\gamma$ 5′ Flanking Region of the Bantu β^{s} Chromosome

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β^{s}-chromosome haplotypes are peculiar to specific regions of Africa and Asia and are associated with the occurrence of different fetal hemoglobin (Hb) levels in sickle cell patients. Among these haplotypes, β^{s}-chromosomes found in the Senegal and the Arab-India regions are associated with relatively high levels of HbF expression, whereas those around the Benin, Bantu, and the Cameroon regions show low levels of HbF expression. The roles of 5′HS2 and the 5′ flanking (promoter region) region in the expression of globin genes are well documented. Haplotype specific variations are found in these regions and have been postulated to be involved in the regulation of HbF expression. In this study, we have analyzed the effect of sequence variations in regulatory regions of the Bantu 5′HS2 and 5′ flanking region of the $^{G}\gamma$ gene on CAT expression. A diminution was observed in K562 cells when the promoter originated from the Bantu β^{s} chromosome. The decreased expression was independent of the origin of the 5′HS2 sequence—combinations of the Bantu promoter were measured with the Benin, Bantu, or Senegal 5′HS2 sequences in K562 cells. However, expression of the same plasmids in murine erythroleukemic (MEL) cells showed no difference in CAT expression among the various sequence combinations studied. Am. J. Hematol. 59:51–56, 1998.

Key words: sickle cell; fetal hemoglobin; Bantu haplotype; LCR 5′HS2, $^{G}\gamma$ 5′ flanking region

INTRODUCTION

Sickle cell anemia is caused by homozygosity for a single point mutation (Glu→Val) at position six of the β-hemoglobin gene [1]. Although there is a single point mutation that results in sickle hemoglobin (Hb), epidemiological studies have concluded that this mutation occurred in Africa in several different locations and on different chromosomes. Malaria, endemic to this region, has acted as the selective force in the expansion of these chromosomes carrying the Glu→Val substitution. Although the disease has a common genetic origin, phenotypic heterogeneity is a common feature of sickle cell disease. Phenotypic heterogeneity associated with sickle cell disease results, in part, from the presence of the sickle mutation on several of these chromosomes that exhibit different patterns of hemoglobin regulation.

The discovery of a polymorphic Hpa-1 restriction site in the β-hemoglobin locus by Kan and Dozy [2] led to the finding of additional polymorphic sites and to the grouping of these polymorphic sequences into specific haplotypes [3,4]. Five major haplotypes are associated with sickle hemoglobin. These have been named according to their region of origin as Benin, Senegal, Bantu, Cameroon, and Arab-India types [5–8]. Each of these haplotypes is associated with variable levels of fetal Hb expression in sickle cell patients. The Benin, Bantu, and Cameroon haplotypes are generally associated with low levels of fetal Hb, whereas the Senegal and Arab-India haplotypes are characterized by high levels of fetal Hb [9–11].

The overall expression of the β-globin gene involves a complex interaction and regulation by multiple factors. A

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partial list includes promoters, enhancers, ubiquitous and erythroid specific transcription factors, as well as the structural configuration of the chromatin [12,13]. Over the last decade, the importance of the β-globin locus control region (LCR) in the regulation and expression of β-globin gene cluster has been well-documented [14–16]. It appears that the complex regulation of globin gene expression is mediated mainly through interactions of the β-like globin gene cluster with the LCR [17]. The LCR, which is located approximately 16 kb 5′ to the ϵ-globin gene, influences gene expression by establishing an active chromatin structure. Position independent expression of a linked gene, and tissue and developmental stage-specific expression of individual genes occurs through interaction of the LCR with specific globin gene promoters and *trans*-acting factors [18,19]. Five erythroid specific DNase 1 hypersensitive sites form the LCR, HS-1,2,3,4 and 5. Of these, only the HS-2 shows significant enhancer activity [20–22]. The HS-2 enhancer activity is localized within a 732 base pair (bp) HindIII-BglII fragment. This region contains binding sites for ubiquitous and erythroid specific factors which include NF-E2/AP-1 and GATA-1 [15]. The HindIII-BglII fragment also contains two tandem AT repeat sequence and several point mutations. This region contains sequence variations that are specific for the various sickle cell chromosomes. Among all the hypersensitive sites tested, haplotype specific mutations are found only in the HS-2 region of the β^s chromosomes [23]. The A→G mutation at position 8,598 of the Benin β^s chromosome enhances the binding of Sp1, whereas the A→T mutation at position 9,114 decreases binding of an ubiquitous *trans*-acting factor present in both HEL and HeLa nuclear extracts [24]. Studies with transgenic mice have shown that a deletion of the Sp1 binding site from 5′HS-2 of the LCR results in a decreased expression of the linked β-globin gene [25]. These findings suggest that mutations in the 5′HS-2 have an important role in the regulation of the fetal Hb gene. These conclusions are further supported by the unusual chromosome arrangement found in a sickle cell patient homozygous for the Benin haplotype and presenting with high levels of fetal hemoglobin. DNA analysis of this patient showed a β^s chromosome that was a hybrid between the LCR HS-2 of Senegal haplotype and approximately 1.3 kb of the 5′ flanking region of the Benin haplotype [23].

The interactions of the LCR 5′HS-2 and the promoter regions of the fetal globin genes are extremely important in the regulation of globin gene expression. The LCR 5′HS-2 and the $^G\gamma$ promoter regions of β^s chromosomes both contain specific sequence variations that correlate with levels of HbF expression. In this study, we have investigated the functional influence of these sequence variations on the expression of the CAT reporter gene in both human (K562) and mouse (MEL) erythroid cells. The results of these experiments show that a marked decrease in expression is associated with the presence of the promoter region of the Bantu β^s chromosome.

TABLE I. Oligonucleotide Primers and Probes Used in the Experiment

Function	Sequence
A) Primers	
$^G\gamma$5′ flanking forward primer	5′-ACGTCATAATCTACCAAGGTCATG-3′
$^G\gamma$5′ flanking reverse primer	5′-GGCGTCTGGACTAGGAGCTTATTG-3′
5′HS-2 forward primer	5′-TAAGCTTCAGTTTTTCCTTAGT-3′
5′HS-2 reverse primer	5′-TAGATCTGACCCCGTATGTGAGCAT-3′
B) Probes	
8580 N	5′-CAGGCCCCGGTCGGGGTC-3′
8580 M	5′-CAGGCCGGTGTCGGGGTC-3′
8598 N	5′-AGTGCCCCACCCCGCCTT-3′
8598 M	5′-AGTGCCCCGCCCCGCCTT-3′
9114 N	5′-CCGTACTTTTGTCCTTTTG-3′
9114 M	5′-GAAAAGGACTAAAGTACGC-3′

MATERIALS AND METHODS

Construction of Expression Vectors

The method of Poncz et al. [26] was used to isolate DNA from the blood of patients homozygous for Senegal, Bantu, and Benin haplotypes. The oligonucleotide primers were used to amplify the 742 bp HindIII-BglII fragment of the LCR HS-2 and the 5′-flanking region of the $^G\gamma$ gene. The sequences of the amplification primers are shown in Table I. The polymerase chain reaction (PCR) was done using a Perkin Elmer DNA Thermal Cycler 480 (Norwalk, CT). The reaction mixture contained 20 mM MOPS, three mM $MgCl_2$, 50 mM KCl, 200 μM of each of the four dNTPs, 1 μg genomic DNA, 50 pg of each of the forward and the reverse primers, and 2.5 U of Taq polymerase. The reaction conditions for the LCR HS-2 fragment for denaturation, annealing, and extension were 94°C for 1.30 min, 58°C for 1.30 min, and 72°C for 3.30 min, respectively. The amplification conditions for the $^G\gamma$ fragment were the same except that the annealing temperature was 56°C. The 1.3 kb flanking region of the $^G\gamma$ gene was amplified using specific oligonucleotide primers containing HindIII recognition sites. The PCR amplified fragments were digested with HindIII and then cloned into the HindIII site of the dephosphorylated pSVOCAT vector. The 5′LCR HS-2 amplified fragments were isolated and purified using Geneclean (BIO 101 Inc., La Jolla, CA). The 742 bp fragments were cloned into the shuttle vector pNOTA using the prime PCR Cloner kit (5Prime-3Prime Inc., Boulder, CO). The HS-2 fragments were excised from the pNOTA by using the BamH1 polylinker site. The purified frag-

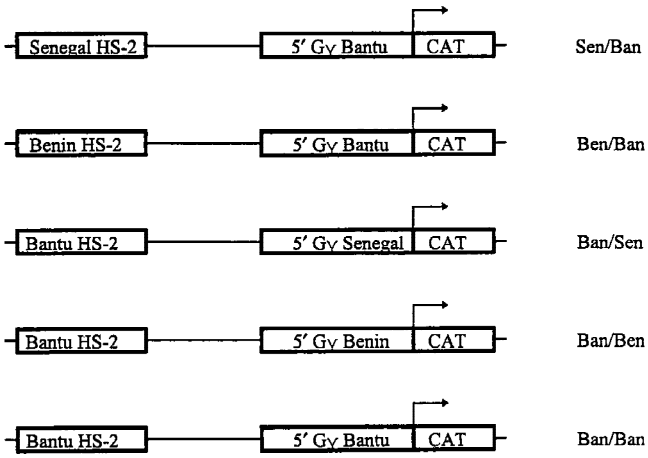

Fig. 1. Globin gene constructs containing the LCR 5′HS-2 and/or the $^{G}\gamma$ 5′ flanking region of the Bantu haplotype.

ments were ligated into the BamH1 site of the dephosphorylated pSVO$^{G}\gamma$CAT expression vector using the Takara ligation kit (Takara Shuzo Co., Kyoto, Japan). All cloned amplified fragments were analyzed by gel electrophoresis following restriction enzyme digestion and by dot blotting to specific probes using the ECL 3′ oligolabelling system (Amersham Life Science, Buckinghamshire, England). The mutant and normal probes used in this experiment are listed in Table I. The nucleotide sequence fidelity of the cloned PCR amplified fragments were determined by DNA sequencing (data not shown). Constructs containing combinations of HS-2 and promoter regions from the various β^{s} chromosomes are shown in Figure 1.

Cell Culture

K562 cells were grown in RPMI 1640 medium and MEL cells were grown in Dulbecco's modified Eagle's medium (Cellgro, Freiburg, Germany) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 10 mM Hepes pH 7.5, 100 U/ml penicillin and 100 μg/ml streptomycin (Mediatech, Herndon, VA). Plasmid DNA was purified for transfection using 2× CsCl gradient centrifugation [27]. Cells were grown to mid-log phase, harvested by centrifugation at 1,000 rpm for five min. Cells were then resuspended in fresh medium to obtain a cell density of 20 million cells/ml, 400 μl of this cell suspension was added to 4 mm BioRad® electroporation cuvettes and mixed with 10 μg of supercoiled test plasmid and 10 μg of pCMVβgal plasmid used as an internal control. The K562 cells were electroporated at 260 V and the MEL cells were electroporated at 220 V with a capacitance at 960uF using the BioRad Gene Pulsar® unit with capacitance extender [28]. After electroporation, cells were transferred immediately into 10 ml of fresh medium and incubated for 48 hr; K562 cells were induced with 500 μM of δ-amino-levulinic acid and the MEL cells were induced with 2% DMSO. Cells were harvested by centrifugation at 1,000 rpm for five min and washed three times with 1× PBS. Cell lysates were prepared by resuspending the cells in 1× reporter lysis buffer (Promega Corp., Madison, WI) followed by three cycles of freeze/thaw; supernatants were collected by centrifugation. The protein concentration in each cell extract was determined using the BCA kit with bovine serum albumin as the standard (Pierce, Rockford, IL).

Reporter Gene Assay

The CAT enzyme assay was done with 20 μg of protein, as described in the manufacturer's protocol (Promega, Madison, WI). C^{14} chloramphenicol (.025 mCi) was purchased from Dupont NEN (Boston, MA). Xylene (Sigma, Inc., St. Louis, MO) was used to extract the product n-butryl C^{14} chloramphenicol from the CAT reaction mixture. The n-butyryl-C^{14} chloramphenicol was then mixed with scintillation fluid (Scintiverse 11, Fisher, Inc., Pittsburgh, PA) and counted in a Beckman LS 6000TA scintillation counter. The β-galactosidase level was measured by the β-galactosidase enzyme assay system essentially as described in the manufacturer's protocol (Promega).

RESULTS

The lowest relative expression was seen with the construct in which the Bantu HS-2 was in combination with the Bantu $^{G}\gamma$ promoter (Figure 2a). The expression was slightly higher but not significantly different from the negative control, pSVOCAT, when the Bantu HS-2 was linked to the promoter regions of either the Senegal or the Benin chromosomes; in both these constructs the CAT expression was 2–3-fold higher. Thus, the Bantu enhancer has the potential to affect a higher level of expression when linked to an appropriate $^{G}\gamma$ promoter sequence. The data also indicate that the extremely low expression of the Bantu enhancer/Bantu promoter combination resulted from the Bantu promoter region and not the 5′HS-2 region. To test this conclusion, constructs that contained the Bantu promoter region in combination with the varied HS-2 regions from the Senegal, Benin, and Bantu chromosomes were transfected into the K562 cells and were analyzed for transient expression of CAT (Figure 2b). The data showed a low level of CAT expression when the Bantu promoter region was present with the Senegal, Benin, or the Bantu HS-2 enhancer fragments. These results indicate that the Bantu 5′ promoter region results in the suppression of the linked reporter gene in the presence of 5′HS-2 fragments from various sickle cell haplotypes.

To ascertain if the observed haplotype related enhancer/promoter effect was developmental stage related, the above constructs were transfected into MEL cells

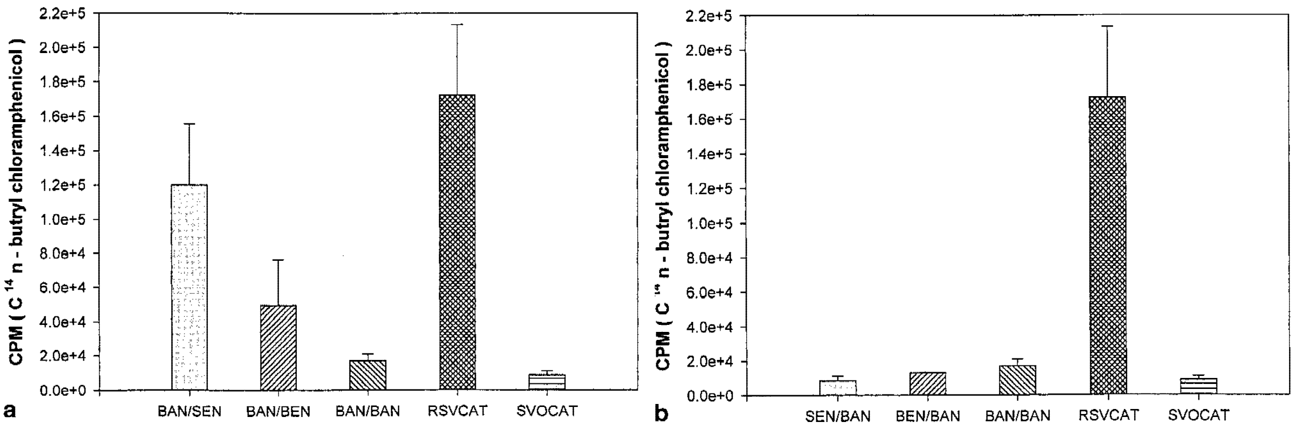

Fig. 2. Effect of the Bantu LCR 5′HS-2 and the $^{G}\gamma$ flanking region on CAT expression in K562 cells; CAT reporter gene assay using C^{14} chloramphenicol and n-butyrl CoA, expressed as CPM of n-butyryl chloramphenicol formed. (a) The 5′ HS-2 of a homozygous Bantu individual in combination with the 5′ flanking $^{G}\gamma$ region of homozygous Senegal, Benin, and Bantu. (b) The $^{G}\gamma$ 5′ flanking region of a homozygous Bantu individual in combination with the LCR HS-2 from the Senegal, Benin, and Bantu. pRSVCAT was used as a positive control and pSVOCAT as the negative control. Each bar represents the mean value with standard deviation from three separate transfections.

(Fig. 3a,b), a cell type with adult Hb phenotype. Interestingly, there was neither a specific inhibition by the Bantu promoter region nor was there a significant difference observed between any of the enhancer/promoter constructs in MEL cells.

DISCUSSION

Recent clinical data have demonstrated multiple factors that affect alterations of HbF levels in sickle cell patients following treatment with hydroxyurea [29]. Patients who have at least one Bantu type β^s chromosome show a reduced response following hydroxyurea treatment. In contrast, patients who have at least one Senegal β^s chromosome respond well to hydroxyurea treatment and show the highest levels of HbF. Patients with the Benin haplotype show a varied response, but in general, the trend is toward the lower end of the spectrum.

The CAT expression data presented here are in agreement with the clinical findings. CAT expression from constructs containing a Bantu promoter in combination with any of the HS-2 fragments from Senegal, Benin, or Bantu β^s chromosomes was low. However, the Senegal promoter fragment in combination with the Bantu HS-2 fragment gave higher levels of expression that approached those of the positive control, pRSVCAT. Thus, the Bantu promoter regions must contain sequences which have an attenuating effect on gene expression in transient promoter/enhancer assays. Benin promoter regions resulted in expression levels that were approximately 50% of those exhibited with the promoter region from the Senegal haplotype. Previous sequencing data have shown the presence of two mutations in the Benin $^{G}\gamma$ promoter region (G at position −369 and −309). The Bantu $^{G}\gamma$ 5′-flanking region has a T at positions −1106 and −1105. In addition, there is a six bp deletion [TTTAAC] located within a tandem repeat between positions −403 to −390 [30,31]. The expression data in Figure 2b suggest that either the six bp deletion or the two T substitutions may play a role in the severely down-regulated gene expression. The conclusion that the six bp deletion is involved in the attenuated gene expression is supported by the finding of a four bp [AGCA] deletion between position −222 to −225, 5′ to the $^{A}\gamma$T allele. This mutation is present in the Cameroon β^s chromosome haplotype. Newborns with this deletion have a significant decrease in total HbF. The lower HbF expression is associated with decreased expression not only of the $^{A}\gamma^{T}$ allele but also the $^{G}\gamma$ gene in *cis* [32,33]. These data support our hypothesis that the decreased expression in the presence of a Bantu β^s haplotype results, in part, from the modulation of the HbF through *cis*-acting elements and *trans*-acting factors. Perhaps the low level of expression with the Bantu promoter is brought about by the disruption of binding of erythroid specific transcription factors.

Expression of the various haplotype enhancer/promoter combinations in MEL cells showed high levels of expression. However, no differences in the relative levels of expression of the reporter gene could be correlated to the haplotype sequence variations. These data are not surprising since it has been shown that MEL cells have an adult phenotype and when transfected with plasmid DNA, fail to show any developmental stage-specific expression of Hb genes [34]. Furthermore, the relative concentrations of various *trans*-acting factors play important roles in the stage-specific expression of the β-globin gene cluster during development [35]. Thus, the

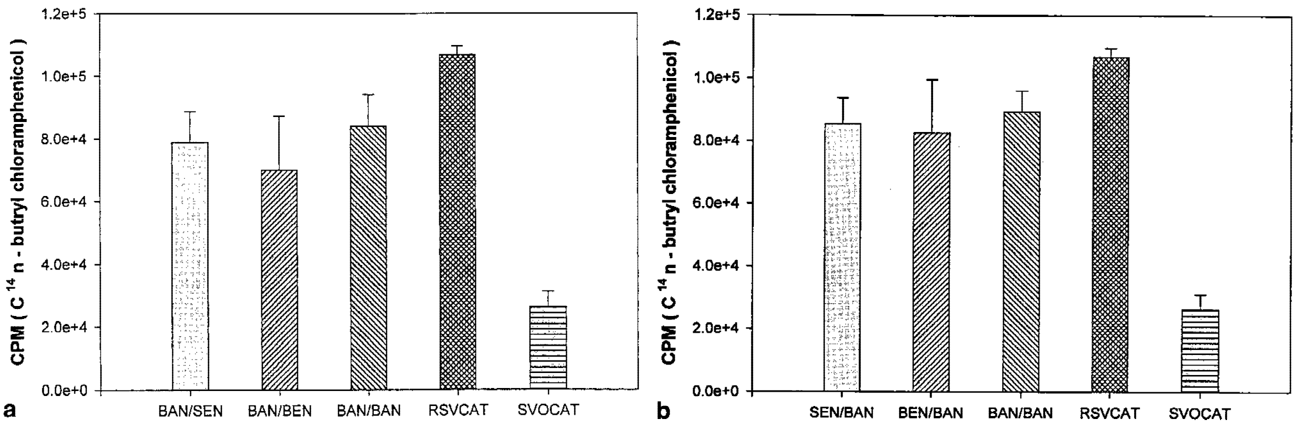

Fig. 3. Effect of the Bantu LCR 5′HS-2 and the $^{G}\gamma$ flanking region on CAT expression in MEL cells; CAT reporter gene assay using C^{14} chloramphenicol and n-butyrl CoA, expressed as CPM of n-butyrl chloramphenicol formed. (a) The 5′HS-2 of a homozygous Bantu individual in combination with the 5′ flanking $^{G}\gamma$ region of homozygous Senegal, Benin, and Bantu. (b) The $^{G}\gamma$ 5′ flanking region of a homozygous Bantu individual in combination with the LCR HS-2 from the Senegal, Benin, and Bantu. pRSVCAT was used as a positive control and pSVOCAT as the negative control. Each bar represents the mean value with standard deviation from three separate transfections.

attenuation of expression that we observed in K562 cells which correlate with sequence variations in promoter region of the Bantu β^s chromosome may reflect both the interaction of and the relative concentrations of specific erythroid and ubiquitous *trans*-acting factors.

Although our expression data reflect the clinical observations regarding individuals with sickle cell anemia and a Bantu haplotype chromosome, these enhancer-promoter interactions are artificial and difficult to interpret with regard to their physiological significance. They clearly represent only part of complex cellular interactions of a number of genetic modulators that lead to HbF induction.

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